

Cell-Cell Propagation of NF- κ B Transcription Factor and MAP Kinase Activation Amplifies Innate Immunity against Bacterial Infection

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SUMMARY

The enteroinvasive bacterium *Shigella flexneri* uses multiple secreted effector proteins to downregulate interleukin-8 (IL-8) expression in infected epithelial cells. Yet, massive IL-8 secretion is observed in Shigellosis. Here we report a host mechanism of cell-cell communication that circumvents the effector proteins and strongly amplifies IL-8 expression during bacterial infection. By monitoring proinflammatory signals at the single-cell level, we found that the activation of the transcription factor NF- κ B and the MAP kinases JNK, ERK, and p38 rapidly propagated from infected to uninfected adjacent cells, leading to IL-8 production by uninfected bystander cells. Bystander IL-8 production was also observed during *Listeria monocytogenes* and *Salmonella typhimurium* infection. This response could be triggered by recognition of peptidoglycan and is mediated by gap junctions. Thus, we have identified a mechanism of cell-cell communication that amplifies innate immunity against bacterial infection by rapidly spreading proinflammatory signals via gap junctions to yet uninfected cells.

INTRODUCTION

The ability of a host organism to mount an innate immune response after pathogen infection is critical for survival. The epithelial cells, which represent the first physical barrier to invasive pathogens, play a critical role in this process. They act as sentinels of the immune system and largely contribute to the secretion of factors that orchestrate inflammation in infected tissues. Infection by *Shigella* bacteria is a well-suited model to analyze the complex host-pathogen interactions that shape the immune response of intestinal epithelial cells (IECs) to invasive bacteria (Phalipon and Sansonetti, 2007). *Shigella* are Gram-negative foodborne bacteria that invade the colonic and rectal epithelium of humans, causing an acute mucosal inflammation called Shigellosis, responsible for 1.1 million deaths annually (Schroeder and Hilbi, 2008).

Shigella flexneri, the etiological agent of the most endemic form of Shigellosis, translocates across the intestinal epithelial barrier by transcytosis through M cells. In the submucosal area, *S. flexneri* makes use of a type III secretion (T3S) apparatus to trigger apoptosis in macrophages and to actively invade IECs via their basolateral surface. The T3S apparatus is a syringe-like nanomachine enabling the translocation of bacterial effector proteins (Cornelis, 2006) that subvert various host cellular pathways in order to promote bacterial entry, modulate cell cycle, and dampen inflammation signaling (Iwai et al., 2007; Parsot, 2009; Phalipon and Sansonetti, 2007). Once internalized, *S. flexneri* multiplies in the cytoplasm and uses actin-based motility to spread to adjacent IECs. During infection, massive mucosal inflammation is observed in the intestine of infected patients (Islam et al., 1997). IECs are critical in this process. They sense pathogenic invasion and respond by inducing a transcriptional program whose major function is to stimulate innate immune defense mechanisms. *Shigella* recognition by IECs occurs essentially intracellularly via the pattern recognition receptor Nod1 that recognizes the core dipeptide structure, γ -D-glutamyl-meso-diaminopimelic acid (Girardin et al., 2003), found in the peptidoglycan of all Gram-negative and certain Gram-positive bacteria (Kufer et al., 2006). Upon ligand recognition, Nod1 homodimerizes and recruits the kinase RIPK2 (Strober et al., 2006). This leads to the sequential recruitment and activation of the TAK1-TAB1-TAB2 and IKK α -IKK β -IKK γ complexes and the phosphorylation of inhibitor of NF- κ B alpha (I κ B α). Once phosphorylated, I κ B α undergoes polyubiquitination and proteasomal degradation. NF- κ B, released from I κ B α , translocates to the nucleus and upregulates expression of proinflammatory genes. TAK1 activation also leads to activation of the MAP kinases c-Jun-N-terminal kinase (JNK) and p38 (Lee et al., 2000; Ninomiya-Tsuji et al., 1999; Wang et al., 2001). The MAP kinase ERK is also activated during *S. flexneri* infection of IECs (Köhler et al., 2002). NF- κ B, JNK, ERK, and p38 contribute collectively to the initiation of a proinflammatory program. JNK and p38 regulate the activity of the transcription factor AP-1 (Holtmann et al., 1999). p38 and ERK control the access of chromatin to transcription factors via phosphorylation of histone H3 by the kinases MSK1 and MSK2 (Saccani et al., 2002). Among the genes upregulated during infection of IECs, the chemokine interleukin-8 (IL-8) plays a central role (Sansonetti et al., 1999) by attracting polymorphonuclear cells (PMNs) from the peripheral circulation to the infected area to limit the spread of *S. flexneri* invasion.

S. flexneri uses multiple strategies to downregulate inflammation. For example, T3 secreted effector proteins OspG and OspF attenuate IL-8 expression by preventing $\text{I}\kappa\text{B}\alpha$ degradation and dephosphorylating nuclear p38 and ERK, respectively (Arbibe et al., 2007; Kim et al., 2005; Li et al., 2007).

Despite the immunosuppressive activity of multiple bacterial effector proteins, massive IL-8 expression is observed in IECs during Shigellosis, suggesting that the secreted molecules may partially block IL-8 expression or that a host mechanism compensates for their effect. Here, we report the discovery of a host cell-cell communication mechanism that circumvents the bacterial effector proteins and amplifies IL-8 expression. By monitoring proinflammatory signals at the single-cell level, we found that, within minutes of infection, the activation of NF- κ B and the MAP kinases JNK, ERK, and p38, propagates from infected to uninfected bystander cells. These cells, in which signaling is not altered by bacterial effector proteins, represent the main source of IL-8 secretion during *S. flexneri* infection. Bystander IL-8 expression can be triggered by recognition of peptidoglycan via Nod1 and is mediated by gap junctions. Thus, we have identified a gap junction-mediated cell-cell communication mechanism that strongly amplifies innate immunity during bacterial infection by rapidly spreading proinflammatory signals to yet-uninfected cells.

RESULTS

NF- κ B Is Activated in Bystander Cells of *S. flexneri* Infection

To better characterize the molecular mechanisms that control inflammation during bacterial infection, we analyzed NF- κ B activation at the single-cell level during *S. flexneri* infection of epithelial cells. The nuclear translocation of the NF- κ B p65 subunit, which follows IKK-mediated $\text{I}\kappa\text{B}\alpha$ degradation, was used as readout for NF- κ B activation and was visualized by immunofluorescence microscopy. HeLa cells were infected with noninvasive BS176 or wild-type invasive M90T *S. flexneri* strains at low and high multiplicity of infection (MOI). As expected, extracellular bacteria failed to activate NF- κ B in HeLa cells as shown by the fact that p65 remained exclusively cytosolic after infection with BS176 *S. flexneri* (Figure 1A, top left). In contrast, cells infected with M90T at MOI 20 showed massive nuclear localization of p65, reflecting the detection of intracellular bacteria and activation of NF- κ B (Figure 1A, top right). Surprisingly, when cells were challenged with M90T at low MOI, a strong p65 nuclear translocation was also observed in some cells that were not infected (Figure 1A, bottom left). Single-cell measurements of *S. flexneri* invasion and p65 nuclear/cytoplasmic ratio (NF- κ B activation ratio) confirmed that, at low MOI, more cells were NF- κ B activated than infected (Figure 1A, bottom right). False color representations of the NF- κ B activation ratio clearly showed that uninfected NF- κ B-activated cells were not randomly distributed in the field of view but located in close proximity with infected cells forming NF- κ B activation foci around them (Figure 1B). A similar pattern of NF- κ B activation was observed during *S. flexneri* infection of the colonic epithelial cell line Caco-2, the lung epithelial cell line A549, and the human umbilical vein endothelial cells (HUVECs) derived from umbilical cord (Figure S1 available

online), suggesting that bystander activation of NF- κ B represents a broad response to *S. flexneri* infection.

During infection, *S. flexneri* uses actin-based motility to spread to adjacent IECs. To control that bystander NF- κ B activation was not due to bacterial intercellular motility, we examined NF- κ B activation in cells infected with the nonmotile *virG* deletion mutant ($\Delta virG$) (Makino et al., 1986). As expected, $\Delta virG$ bacteria efficiently invaded HeLa cells (Figure 1C, right). However, unlike wild-type (Figure 1C, left), they failed to form actin comet tails and accumulated overtime in the perinuclear region (Figure 1C, right). Interestingly, infection with the $\Delta virG$ mutant induced p65 nuclear translocation in infected and in bystander cells (Figure 1D) similarly to infection with wild-type *S. flexneri* (Figure 1A), suggesting that bystander NF- κ B activation was not caused by intercellular motility, but reflected instead a novel host response to bacterial infection. Because the intracellular microcolonies formed by the $\Delta virG$ mutant were easily detected by automated image analysis, this mutant was used hereafter in our studies.

In order to further characterize bystander NF- κ B activation, we analyzed its kinetic during *S. flexneri* infection in HeLa cells. Representative examples of bystander activation at different time points were chosen for illustration (Figure 1E). Within 15 min of infection, NF- κ B was almost exclusively activated in infected cells. Then, at 30 min and up to 120 min, NF- κ B activation was observed in infected and bystander cells, suggesting that the signals underlying bystander NF- κ B activation were generated very early during infection (within 30 min) and propagated from infected to bystander cells. Considering that each infected cell was surrounded by approximately 2–6 NF- κ B-activated cells, our results demonstrate that this mechanism of cell-cell communication strongly amplifies the total NF- κ B response to *S. flexneri* infection.

JNK and ERK Are Also Activated in Bystander Cells of *S. flexneri* Infection

The activation of the JNK signaling pathway is required to mount an inflammatory response, and in particular, to induce IL-8 expression. We therefore tested, whether in addition to NF- κ B, the JNK pathway was also activated in bystander cells of *S. flexneri* infection in HeLa and Caco-2 cells. JNK activation was analyzed by immunofluorescence microscopy via a phospho-specific antibody that detects p46 and p54 JNKs phosphorylated at residues threonine 183 and tyrosine 185 (p-JNK). Cytosolic p65 localization and basal p-JNK staining indicated that both pathways were inactive in control cells (Figure 2A). As expected, a clear nuclear translocation of p65 and a significant increase of p-JNK were observed in *S. flexneri*-infected cells. Interestingly, bystander cells of infection also showed an increase of p-JNK, indicating that the JNK pathway was turned on in these cells as well (Figure 2A and Figure S2A). This observation was confirmed by measuring with automated image processing the degree of nuclear p-JNK in control, infected, and bystander cell populations (Figure 2B, see Supplemental Experimental Procedures).

In addition to JNK, the activation of ERK was analyzed by immunofluorescence microscopy by means of an antibody that recognizes p22 and p44 ERKs phosphorylated at residues threonine 202 and tyrosine 204 (p-ERK). Representative images of NF- κ B and p-ERK at different time points were chosen for

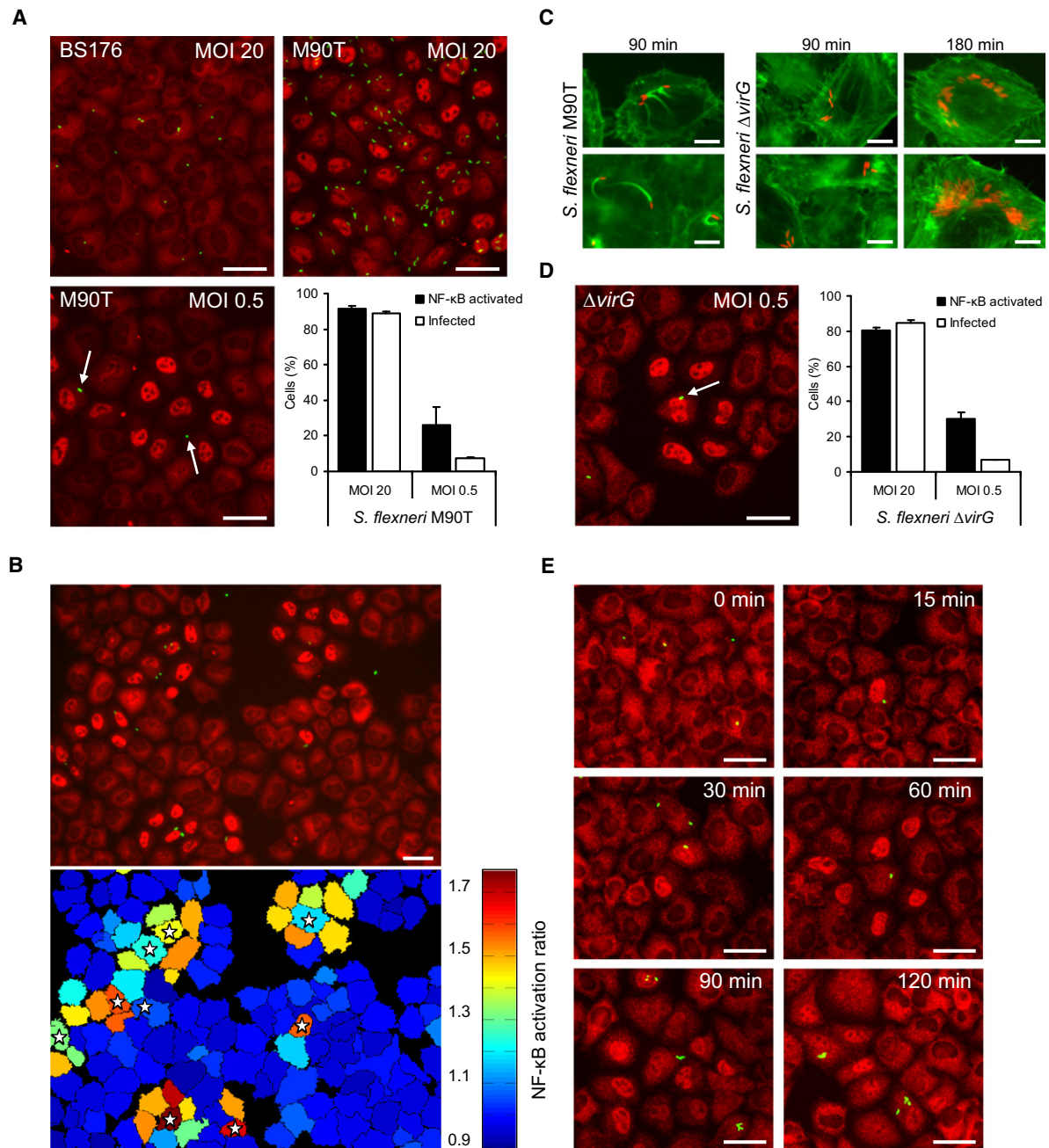


Figure 1. NF-κB Is Activated in Bystander Cells of *S. flexneri* Infection

(A) Nuclear localization of NF-κB p65 during *S. flexneri* infection. HeLa cells were infected for 1 hr with BS176 (MOI = 20, top left) and M90T (MOI = 20, top right; MOI = 0.5, bottom left), stained with a p65 antibody, and visualized by fluorescence microscopy (p65 in red, *S. flexneri* in green). Arrows indicate *S. flexneri*. Percent of NF-κB activated and infected cells at high and low MOI (bottom right, means \pm SD of triplicate wells, graph representative of two independent experiments). Scale bars represent 40 μ m.

(B) False color representation of NF-κB activation ratio during *S. flexneri* M90T infection of HeLa cells. Stars indicate infected cells. Scale bar represents 40 μ m.

(C) Actin-based motility of *S. flexneri* M90T (left) and Δ*virG* mutant (right). After infection, cells were stained for F-actin with phalloidin (F-actin in green, *S. flexneri* in red). Scale bars represent 10 μ m.

(D) Bystander NF-κB activation during infection with *S. flexneri* Δ*virG* (left). p65 in red, *S. flexneri* in green. Arrow indicates *S. flexneri*. Percent of NF-κB activated and infected cells at high and low MOI (right, means \pm SD of triplicate wells, graph representative of two independent experiments). Scale bar represents 40 μ m.

(E) Time course of p65 translocation during infection with *S. flexneri* Δ*virG*. Representative images were selected for illustration. Scale bars represent 40 μ m.

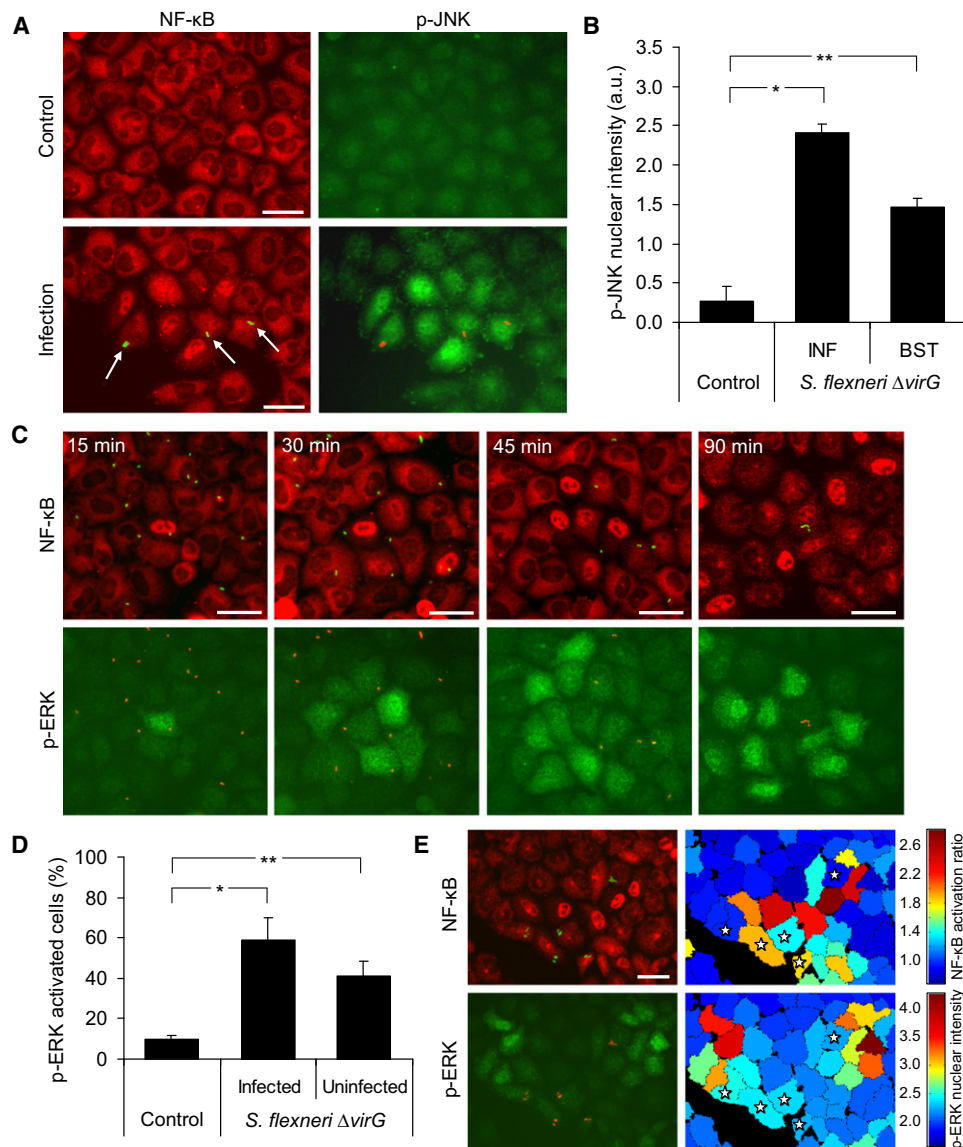


Figure 2. JNK and ERK Are Activated in Bystander Cells of *S. flexneri* Infection

(A) Analysis of JNK and NF-κB activation by immunofluorescence microscopy. HeLa cells were left untreated or infected with *S. flexneri* $\Delta virG$ at MOI = 0.5 for 90 min and costained with p65 and p-JNK antibodies. Arrows indicate *S. flexneri*. Scale bars represent 40 μ m.

(B) Quantification of nuclear p-JNK intensity in control, infected (INF), and bystander (BST) cell populations by automated image processing (a.u., arbitrary units, means \pm SD of triplicate wells, graph representative of five independent experiments, * p = 7.2E-5, ** p = 7.6E-4).

(C) Time course of ERK and NF-κB activation during infection with *S. flexneri*. HeLa cells were infected with *S. flexneri* $\Delta virG$ at MOI = 0.5 for indicated time periods and costained with p65 and p-ERK antibodies. Scale bars represent 40 μ m.

(D) Percent of ERK-activated cells during *S. flexneri* infection. Cells were infected for 1 hr at MOI = 5. Quantification was performed by automated image processing as described in [Supplemental Experimental Procedures](#) (means \pm SD of triplicate wells, graph representative of three independent experiments, * p = 1.9E-3, ** p = 4.7E-3).

(E) False color representations of NF-κB activation ratio and nuclear p-ERK 1 hr after infection with *S. flexneri* $\Delta virG$. Stars indicate infected cells. Scale bar represents 40 μ m.

illustration (Figure 2C). Within 15 min of infection, ERK activation was observed in only a fraction of infected cells. At 30 min and up to 90 min, ERK was also activated in bystander cells of infection (Figures 2C and 2D and Figure S2B). NF-κB and ERK activation did not strictly correlate at the single-cell level. Indeed, after 45 min of infection, ERK activation was no longer visible in a fraction of infected or proximal bystander cells, suggesting that this

pathway was only transiently induced. Furthermore, ERK activation was observed in cells located outside the NF-κB activation foci, suggesting that ERK activation preceded NF-κB activation (Figures 2C and 2E). Altogether, these results suggested that besides the activation of NF-κB, the activation of JNK and ERK also propagates from infected to bystander cells during *S. flexneri* infection of HeLa and Caco-2 cells.

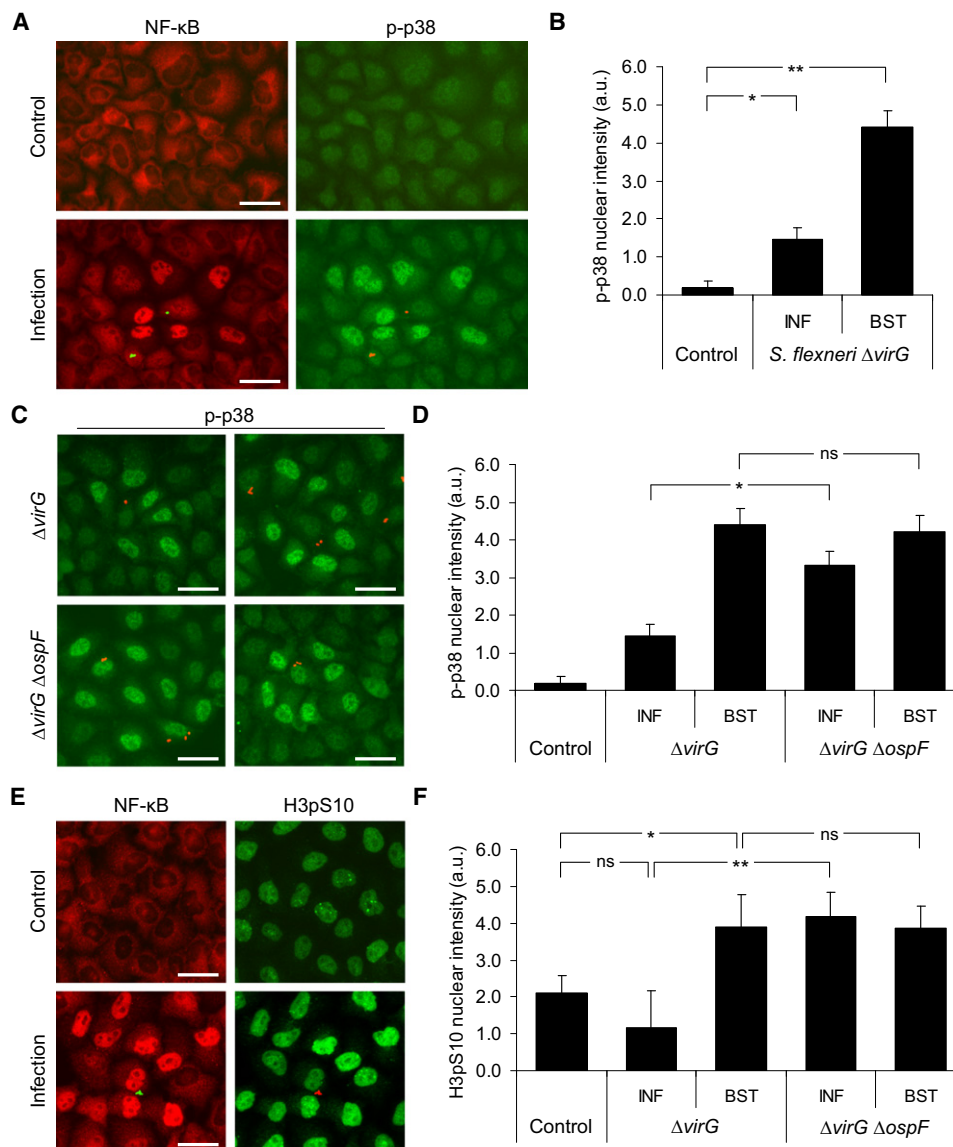


Figure 3. p38 Activation and Histone H3 Phosphorylation Occur Mainly in Bystander Cells

(A) Analysis of p38 and NF- κ B activation by immunofluorescence microscopy. HeLa cells were left untreated or infected with *S. flexneri* $\Delta virG$ at MOI = 0.5 for 90 min and costained with p65 and p-p38 antibodies. Scale bars represent 40 μ m.

(B) Quantification of nuclear p-p38 intensity in control, infected (INF), and bystander (BST) cell populations (a.u., arbitrary units, means \pm SD of triplicate wells, graph representative of five independent experiments, *p = 4.1E-3, **p = 1.0E-4).

(C) Phosphorylation of p38 in cells infected with $\Delta virG$ or $\Delta virG \Delta ospF *S. flexneri* visualized by immunofluorescence microscopy. Scale bars represent 40 μ m.$

(D) Quantification of nuclear p-p38 intensity by automated image processing in control, infected, and bystander cell populations during infection with $\Delta virG$ or $\Delta virG \Delta ospF *S. flexneri* (means \pm SD of triplicate wells, graph representative of five independent experiments, *p = 4.2E-3).$

(E) Analysis of histone H3 phosphorylation by immunofluorescence microscopy. HeLa cells were left untreated or infected with *S. flexneri* $\Delta virG$ at MOI = 0.5 for 90 min and costained with p65 and H3pS10 antibodies. Scale bars represent 40 μ m.

(F) Quantification of nuclear H3pS10 intensity in control, infected, and bystander cell populations (means \pm SD of triplicate wells, graph representative of five independent experiments, *p = 4.9E-2, **p = 1.1E-3).

p38 Activation and Histone H3 Phosphorylation Mainly Occur in Bystander Cells of Infection

Because p38 activation is also critical for IL-8 expression, we examined whether p38 was activated in bystander cells of *S. flexneri* infection in HeLa and Caco-2 cells. p38 activation was analyzed by immunofluorescence microscopy by means of an antibody that detects p38 phosphorylated at residues three-

online 180 and tyrosine 182 (p-p38). As reported previously (Arbibe et al., 2007), a very modest increase of p38 activation was observed in infected cells (Figures 3A and 3B and Figure S3). In contrast, a strong increase was found in bystander cells of infection indicating that, in addition to NF- κ B, JNK, and ERK, p38 was also activated in these cells (Figures 3A and 3B and Figure S3). Signal transduction in infected cells is altered by multiple

effectors that translocate into the host cytoplasm via the T3S apparatus. In particular, p38 is dephosphorylated in the nucleus of infected cells by the phosphothreonine-lyase activity of OspF (Li et al., 2007). Given that bystander cells showed massive p38 activation, we hypothesized that the activation of p38 in bystander cells was not affected by OspF. To test this assumption, HeLa cells were infected with $\Delta virG$ and $\Delta virG \Delta ospF$ *S. flexneri* mutants. As previously reported (Arbibe et al., 2007), p38 activation was restored in cells infected with $\Delta ospF$ bacteria, confirming the role of OspF in p38 dephosphorylation (Figures 3C and 3D). In contrast, the amount of p38 activation in bystander cells remained unchanged (Figure 3D), indicating that OspF failed to impair the ability of the host to spread p38 activation to neighboring cells. Taken together, our data showed that the mechanism of bystander p38 activation circumvents the suppressive activity of OspF in infected cells and amplifies p38 activation during *S. flexneri* infection.

In addition to its role in AP-1 phosphorylation, p38 controls IL-8 expression by regulating chromatin accessibility to transcription factors such as NF- κ B via the phosphorylation of histone H3 by MSK1 and MSK2 (Saccani et al., 2002). To assess whether bystander p38 activation led to histone H3 phosphorylation in bystander cells, phosphorylation at serine 10 (H3pS10) was analyzed by immunofluorescence microscopy via a phospho-specific antibody. To minimize H3pS10 staining from mitotic cells, HeLa cells were arrested in S phase by a double-thymidine block. Consistent with the pattern of p38 activation during *S. flexneri* infection, H3pS10 was higher in bystander than infected cells (Figures 3E and 3F). Furthermore, the deletion of *ospF* restored H3pS10 in infected cells but had no effect in bystander cells (Figure 3F). Collectively, these results demonstrate that p38 activation and the subsequent phosphorylation of histone H3, which are both impaired in infected cells because of the activity of OspF, are fully operating in bystander cells of *S. flexneri* infection.

Cell-Cell Propagation of Proinflammatory Signals Amplifies Cytokine Expression

The NF- κ B, JNK, ERK, and p38 signaling pathways were turned on in bystander cells of *S. flexneri* infection. Given that these pathways control the expression of proinflammatory genes including IL-8, we tested whether bystander cells of *S. flexneri* infection secreted IL-8. IL-8 secretion was first measured by an enzyme-linked immunosorbent assay (ELISA) in the supernatant of HeLa cells infected by *S. flexneri* at different MOIs. We observed that the amount of secreted IL-8 decreased as the MOI was raised (Figure 4A). Because low and high MOIs corresponded to low and high infected to bystander cell ratios, respectively (Figure S4A), this result suggested that IL-8 was most probably secreted by bystander cells of infection. This hypothesis was tested by in situ mRNA hybridization to visualize at the single-cell level the amount of IL-8 mRNA produced during *S. flexneri* infection. As shown in Figure 4B, IL-8 mRNAs were almost exclusively present in bystander cells of infection. To confirm that bystander cells were the main IL-8-producing cells during *S. flexneri* infection, we performed an intracellular IL-8 immunofluorescence microscopy assay in cells treated with monensin, a protein transport inhibitor that blocks secretion and enables intracellular IL-8 accumulation in the Golgi appa-

ratus (Mollenhauer et al., 1990). In line with the ELISA and the mRNA data, almost no IL-8 was visible in infected cells, but massive intracellular IL-8 accumulation was found in bystander cells (Figures 4C and 4D). An average of 2.8 ± 1.6 , 4.2 ± 2.5 , 6.5 ± 4.7 , and 29.3 ± 13.5 bystander IL-8-producing cells per infected one was measured in HeLa, Caco-2, A549, and HUVEC cells, respectively (Figures S4B–S4D). Interestingly, tumor necrosis factor α (TNF- α) and granulocyte macrophage colony-stimulating factor (GM-CSF), two other inflammatory cytokines upregulated during *S. flexneri* infection of epithelial cells (Pédrón et al., 2003), were also found in bystander cells (Figures S4E–S4G), suggesting that the mechanism of bystander activation contributes to different facets of inflammation during infection.

Strong cytokine expression in bystander cells indicated that cell-cell communication was not affected by *S. flexneri* effector proteins. This was tested by investigating the effect of OspF on IL-8 expression in infected and bystander cell populations. As previously reported (Arbibe et al., 2007), an increase of IL-8 production was observed in cells infected with $\Delta ospF$ *S. flexneri* (Figure 4E). In contrast, the number of bystander cells producing IL-8 was independent of OspF (Figure 4F) indicating that, in line with our p38 activation and H3pS10 results, OspF failed to affect IL-8 expression in bystander cells.

To further characterize the mechanism of bystander activation, we tested whether it also occurred after infection with *Listeria monocytogenes* or *Salmonella typhimurium*, two enteroinvasive bacteria that induce IL-8 expression during invasion of IECs (Eckmann et al., 1993). Consistent with data on *S. flexneri* infection, IL-8 accumulation was also observed in bystander cells of *L. monocytogenes* and *S. typhimurium* infection (Figure 4G) but at a lower frequency. In conditions where bystander IL-8 expression was observed for nearly 100% of *S. flexneri*-infected cells, it occurred for approximately 40% and 70% of *Listeria*- and *Salmonella*-infected cells, respectively. In contrast to *S. flexneri* infection, IL-8 accumulation was also detected in a fraction of cells infected with *L. monocytogenes* or *S. typhimurium* (approximately 20% and 40%, respectively), indicating that these bacteria do not manipulate their host to the same extent as *S. flexneri*, for which low levels of IL-8 were detected in less than 5% of infected cells (Figure 4E). Altogether these results show that cell-cell communication between infected and uninfected bystander cells leads to the potentiation of inflammatory cytokine expression during bacterial infection. They also establish that this is a general host response to invasive bacteria that occurs with an amplitude and a frequency that vary between cell types and pathogens.

Nod1-Mediated Peptidoglycan Sensing Is Sufficient to Induce Bystander IL-8 Expression

During *S. flexneri* infection, the presence of intracellular bacteria is sensed through peptidoglycan recognition by the intracellular receptor Nod1 (Girardin et al., 2003). To determine whether pathogen sensing via Nod1 was sufficient to induce bystander IL-8 production, we monitored IL-8 accumulation after microinjection of the synthetic Nod1 ligand L-Ala-D- γ -Glu-meso-diaminopimelic acid (TriDAP) into the cytoplasm of A549 cells. An Alexa 488-labeled IgG antibody (IgG A488) was used as fluorescent marker to identify microinjected cells. In response to IgG A488 microinjection, no IL-8 was detected (Figure 5A, left, and

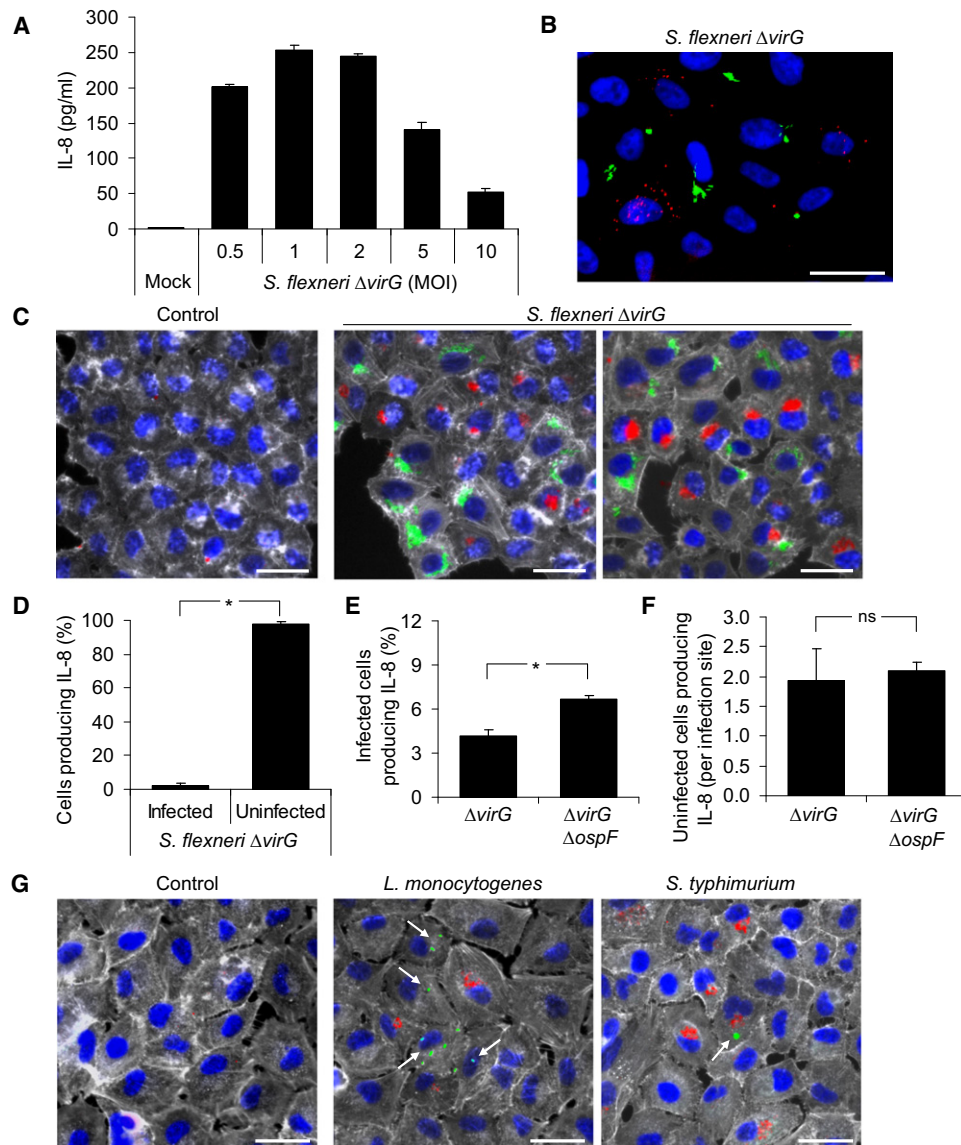


Figure 4. IL-8 Production by Bystander Cells Is a General Response to Bacterial Infection

(A) Measurements of IL-8 secretion by ELISA 6 hr after infection (means \pm SD of triplicate wells, graph representative of two independent experiments).
 (B) Visualization of IL-8 mRNA 2 hr after infection by in situ hybridization (IL-8 mRNAs in red, *S. flexneri* in green, Hoechst in blue, MOI = 2). Scale bar represents 40 μ m.
 (C) IL-8 accumulation in bystander cells of infection. IL-8 staining of monensin-treated HeLa cells 3 hr after infection (IL-8 in red, *S. flexneri* in green, Hoechst in blue, F-actin in gray, MOI = 2). Scale bars represent 40 μ m.
 (D) Percent of infected and uninfected cells among all IL-8-producing cells (MOI = 1). Quantification was performed by automated image processing based on the use of threshold intensity values for bacterial and IL-8 detection (means \pm SD of triplicate wells, graph representative of three independent experiments, $^*p = 3.9E-16$).
 (E) Percent of infected cells producing IL-8 during infection with $\Delta virG$ and $\Delta virG \Delta ospF$ *S. flexneri* (MOI = 5). Quantification was performed as described in (D) (means \pm SD of triplicate wells, graph representative of two independent experiments, $^*p = 4.5E-4$).
 (F) Number of uninfected cells producing IL-8 per site of infection during infection with $\Delta virG$ and $\Delta virG \Delta ospF$ *S. flexneri* (MOI = 1). Quantification was performed as described in (D) (means \pm SD of triplicate wells, graph representative of two independent experiments).
 (G) IL-8 production (in red) in bystander cells during *L. monocytogenes* (MOI = 0.25, green) and *S. typhimurium* (MOI = 0.5, green) infection of A549 cells. F-actin in gray, arrows indicate bacteria. Scale bars represent 40 μ m.

Figure 5B). In contrast, when TriDAP was combined with IgG A488, both microinjected and bystander cells showed massive intracellular IL-8 accumulation (Figure 5A, right, and Figure 5B). To verify that IL-8 production was not caused by extracellular

TriDAP leaking during microinjection, the concentration of TriDAP used in the microcapillary was uniformly applied to the extracellular medium. In contrast to TNF- α , extracellular TriDAP failed to induce IL-8 expression (Figure 5C and Figure S5).

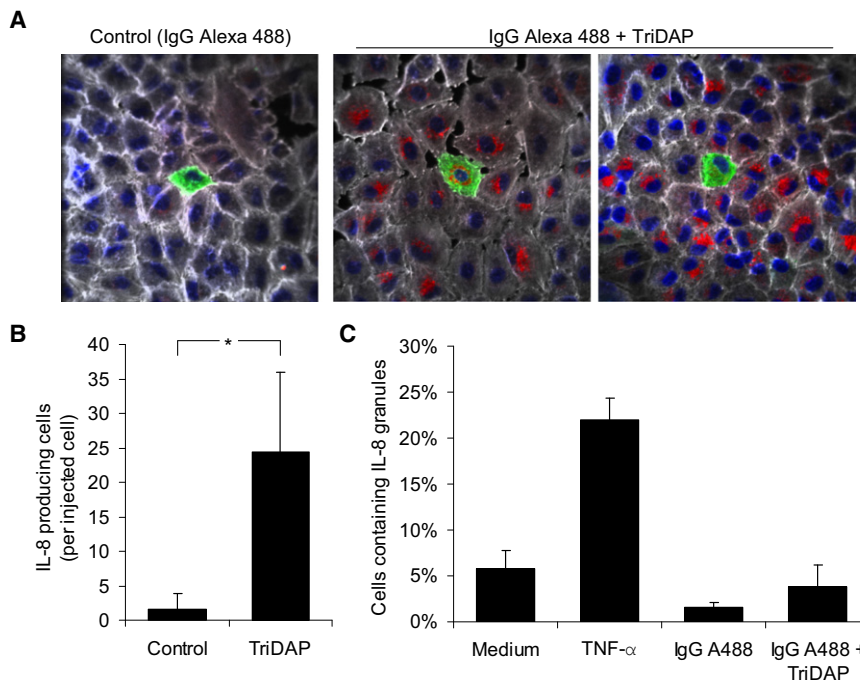


Figure 5. Intracellular Recognition of a Nod1 Ligand Is Sufficient to Induce IL-8 Expression in Bystander Cells

(A) IL-8 accumulation in bystanders of microinjected A549 cells. After injection of IgG Alexa 488 alone (left) or combined with TriDAP (right), cells were stained for IL-8, F-actin, and DNA (IgG A488 in green, IL-8 in red, F-actin in gray, Hoechst in blue).

(B) Number of IL-8-producing cells per injected cell. Quantification was performed by counting all IL-8-expressing cells located in contact with microinjected cells or other bystander cells (control, $n = 20$; TriDAP, $n = 21$; $*p = 1.0E-9$).

(C) Percent of cells containing IL-8 after extracellular treatment with TNF- α , IgG A488 alone, or combined with TriDAP. Cells were stained for IL-8 and DNA and analyzed by automated image processing (means \pm SD of triplicate wells, graph representative of two independent experiments).

Together, these results suggested that Nod1-mediated recognition of intracellular TriDAP was necessary and sufficient to induce IL-8 expression by bystander cells of microinjection. Because Nod1-mediated recognition of peptidoglycan also takes place in *S. flexneri*-infected cells, this result suggested that pathogen sensing may be sufficient to trigger IL-8 expression in bystander cells of *S. flexneri* infection.

Bystander Activation Is Not Mediated by Paracrine Signaling but Requires Cell-Cell Contact

Reports indicating that TNF- α is upregulated during *S. flexneri* infection and that NF- κ B, p38, ERK, and JNK are activated by TNF- α suggested that this cytokine may induce bystander activation via paracrine signaling (Dong et al., 2002; Pédrón et al., 2003). This hypothesis was tested by examining bystander activation in tumor necrosis factor receptor 1 (TNFR1)-deficient mouse embryonic fibroblasts (*Tnfr1*^{-/-} MEFs). Because mice are deficient for the *IL8* gene, the chemokine macrophage inflammatory protein-2 (MIP-2) was chosen as readout of inflammation. Whereas *Tnfr1*^{-/-} cells did not respond to TNF- α stimulation, massive MIP-2 expression was observed in bystander cells of *S. flexneri* infection (Figure 6A; Figures S6A and S6B), indicating that TNF- α was not the mediator of bystander activation. To investigate the role of protein secretion more broadly, we tested whether this process was impaired when protein secretion was abolished by the protein transport inhibitor brefeldin A (BFA). For conditions of drug treatment that blocked phorbol 12-myristate 13-acetate (PMA)-induced IL-8 secretion (Figure S6C), BFA had no effect on bystander activation during *S. flexneri* infection of Caco-2 cells (Figures 6B and 6C), suggesting that cell-cell propagation of proinflammatory signals was not mediated by secreted proteins.

To further explore the hypothesis of paracrine signaling, experiments of *S. flexneri* infection were performed in a flow chamber where fresh medium was perfused to wash away any potential secreted factors. A flow rate corresponding to the replacement of the entire volume of the chamber every second was used. IL-8 accumulation was still visible in bystander cells of infection located along an axis perpendicular or opposite to the direction of the flow (Figure S6D). Quantification of IL-8 (Figure 6D) showed no effect of perfusion, indicating that bystander activation was very improbably mediated by a long-ranged diffusing soluble factor.

To characterize the mechanism of bystander activation, we investigated whether it was cell-cell contact dependent. Infection was performed at subconfluent cell density to evaluate IL-8 expression in Caco-2 cells that had no physical interactions with infected cells. Inspection of images and manual quantification indicated that IL-8 was exclusively found in cells having direct or indirect contacts with infected cells and defined as class 1 (Figures 6E and 6F). Class 2 cells present in the vicinity of the infected cell (Experimental Procedures) but, separated by a gap, did not exhibit markedly more IL-8 than class 3 cells distant from any infection foci (Figure 6F). Collectively, these results demonstrated that the expression of IL-8 in bystander cells depends on cell-cell contact and is most probably not mediated by paracrine signaling.

Cell-Cell Propagation of Inflammatory Signals Is Mediated by Connexin Gap Junctions

An alternative hypothesis to paracrine signaling is direct communication via gap junction channels formed by connexin proteins. This hypothesis was directly tested by evaluating the effect of the gap junction blocker 18 β -glycyrrhetic acid (18 β -GA) on IL-8 expression in bystander cells during *S. flexneri* infection of Caco-2 cells. In conditions of drug treatment that blocked Lucifer Yellow transfer through gap junctions of adjacent Caco-2 cells (Figure S7A), IL-8 expression in bystander

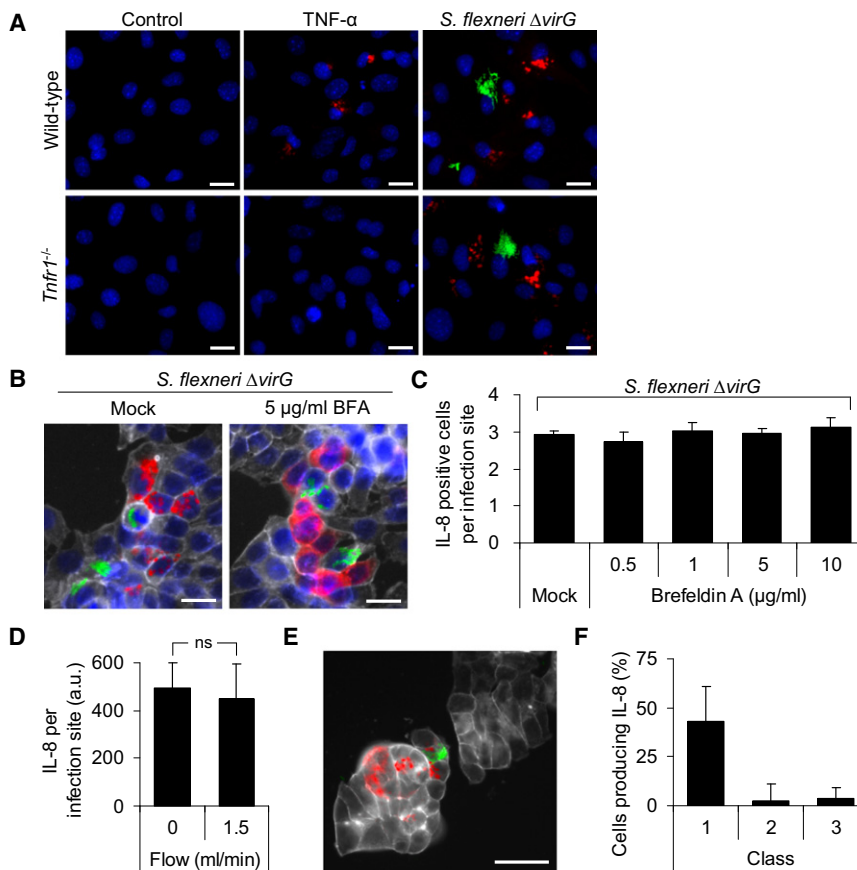


Figure 6. Bystander Activation Is Not Mediated by Paracrine Signaling but Is Cell-Cell Contact Dependent

(A) MIP-2 expression in wild-type and *Tnfr1*^{-/-} MEF cells after TNF- α stimulation or *S. flexneri* infection visualized by fluorescence microscopy. After infection, cells were stained for MIP-2 and DNA with a MIP-2 antibody and Hoechst, respectively (MIP-2 in red, *S. flexneri* in green, Hoechst in blue). Scale bars represent 20 μ m.

(B) Bystander IL-8 expression in Caco-2 cells pre-treated with BFA and infected with *S. flexneri*. After infection (*S. flexneri* in green), cells were stained for IL-8, DNA, and F-actin with an IL-8 antibody (in red), Hoechst (in blue), and phalloidin (in gray), respectively. Scale bars represent 20 μ m.

(C) Quantification of bystander IL-8 expression upon BFA treatment by automated image analysis (means \pm SD of triplicate wells, graph representative of two independent experiments).

(D) Quantification of bystander IL-8 expression under flow conditions. IL-8 was quantified by measuring for each infected cell the area of IL-8 staining (a.u., arbitrary units, means \pm SD, $n = 10$, graph representative of two independent experiments).

(E) Cell-cell contact analysis of bystander IL-8 expression. Caco-2 cells, seeded at subconfluent density and infected with *S. flexneri* (in green), were stained for IL-8 (in red) and F-actin (in gray). Scale bar represents 50 μ m.

(F) Fractions of IL-8-producing cells in class 1, 2, 3 cell populations as defined in [Experimental Procedures](#) (means \pm SD, $n > 38$, graph representative of two independent experiments).

cells of infection was strongly reduced (Figures 7A and 7B). In contrast, TNF- α -induced IL-8 secretion, used as control, was not affected (Figures S7B and S7C). A similar result was obtained with the gap junction inhibitor carbenoxolone (Figures S7D and S7E). Furthermore, treatment with glycyrrhizic acid, a compound structurally related to 18 β -GA but which fails to block gap junction communication at concentrations below 100 μ M (Davidson et al., 1986), had no effect on *S. flexneri*-induced IL-8 expression by bystander cells (Figure 7B). Taken together, these results suggested that IL-8 expression by bystander cells of infection was mediated by communication through gap junctions.

Because gap junction inhibitors have unspecific effects, we further validated this finding by testing whether the propagation of inflammatory signals was connexin dependent. In A431 cells that are poorly coupled via gap junctions and express connexin43 (Cx43) below the level of detection with antibodies (Trojanovsky et al., 1994), very limited activation of NF- κ B, JNK, p38, and ERK and residual IL-8 expression were found in bystander cells of *S. flexneri* infection (Figure 7C, left, and Figure 7D, top). In contrast, in Cx43-overexpressing A431 cells (A431-Cx43) that are effectively coupled via gap junctions (Neijssen et al., 2005), large foci of NF- κ B, JNK, p38, and ERK activation and IL-8 expression were found around infected cells (Figure 7C, right, and Figure 7D, bottom). Quantification of IL-8 expression in consecutive A431 or A431-Cx43 bystander cells

by automated image processing, as described in [Supplemental Experimental Procedures](#), confirmed that the propagation of IL-8 expression was Cx43 dependent (Figures 7D and 7E). As expected, bystander IL-8 expression was strongly reduced when A431-Cx43 cells were depleted of Cx43 by RNA interference (Figure 7F and Figure S7F) or treated with 18 β -GA (Figure 7G and Figure S7G).

In contrast to typical hemichannel-based signaling, communication via gap junction channels requires connexin proteins in both donor and recipient cells. To confirm that this condition was fulfilled for bystander activation, the propagation of inflammatory signals from *S. flexneri*-infected A431-Cx43 cells to either A431-Cx43 or A431 bystander cells was analyzed in experiments where A431-Cx43 and A431 cells were mixed prior seeding. Cx43, used to discriminate A431 and A431-Cx43 cells, as well as IL-8 were detected by immunofluorescence. Whereas IL-8 expression robustly spread within consecutive A431-Cx43 cells, the propagation from infected A431-Cx43 to adjacent A431 cells was very limited (Figure S7H). This observation, quantified by automated image processing (Figure 7H and Figure S7I), indicated that Cx43 proteins were also required in uninfected bystander cells to efficiently potentiate inflammation. Taken together, these data convincingly showed that the propagation of inflammation during bacterial infection of epithelial cells depends on connexin gap junctions.

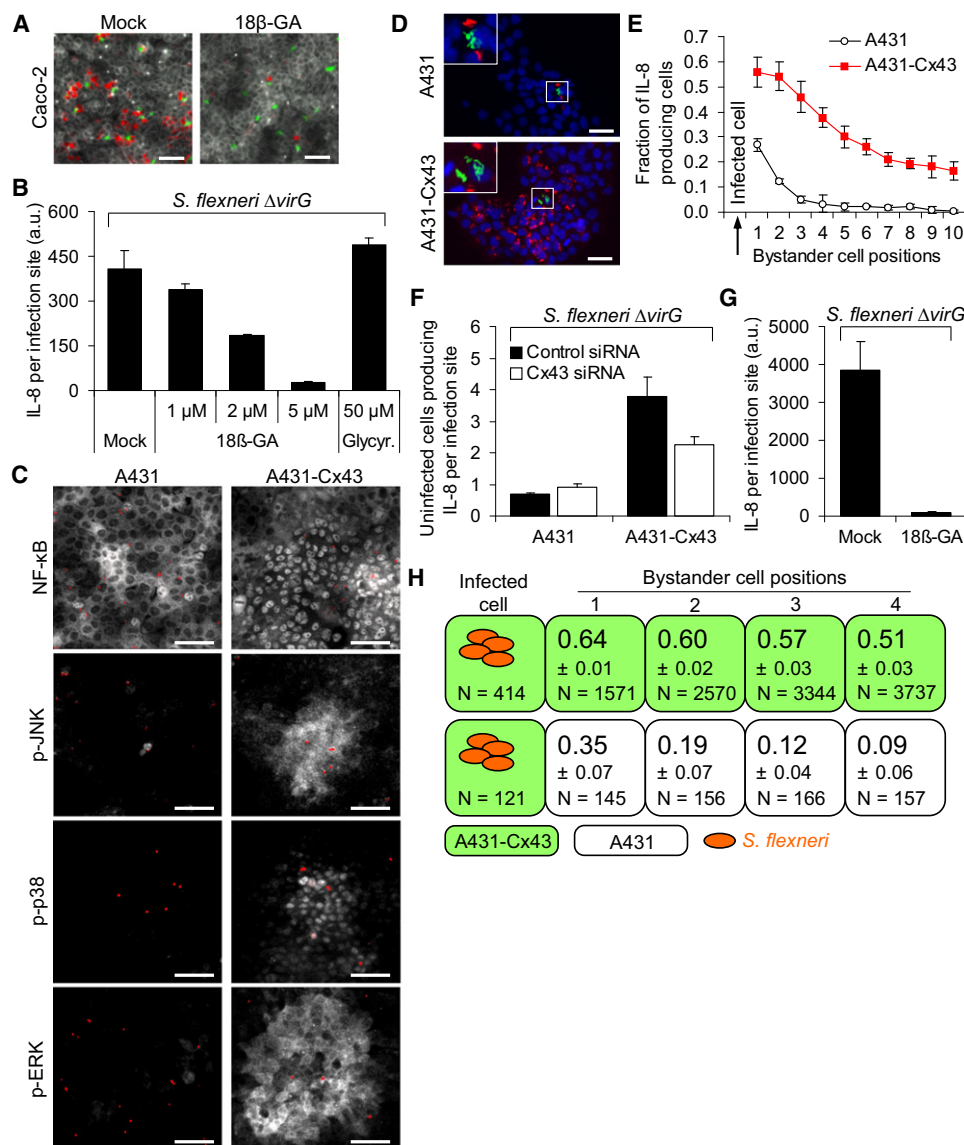


Figure 7. Propagation of Inflammation Is Mediated by Gap Junctions

(A) Effect of 18β-GA on IL-8 expression during infection of Caco-2 cells. Cells were pretreated with 18β-GA, infected with Δ*virG* *S. flexneri*, and stained for IL-8 and F-actin with an IL-8 antibody and phalloidin, respectively (IL-8 in red, *S. flexneri* in green, F-actin in gray). Scale bars represent 50 μm.

(B) Quantification of IL-8 accumulation per site of infection in cells left untreated or pretreated with 18β-GA or glycyrrhizine. Quantification was performed by automated image processing as described in [Supplemental Experimental Procedures](#) (means ± SD of triplicate wells, graph representative of two independent experiments).

(C) Cell-cell propagation of proinflammatory signals in A431 and A431-Cx43 cells visualized by immunofluorescence. NF-κB p65, p-JNK, p-p38, and p-ERK immunofluorescence staining in A431 cells (left) and A431-Cx43 cells (right) after *S. flexneri* infection (NF-κB p65, p-JNK, p-p38, and p-ERK in gray, *S. flexneri* in red). Scale bars represent 40 μm.

(D) Propagation of IL-8 expression during *S. flexneri* infection of A431 and A431-Cx43 cells visualized by immunofluorescence. After infection, cells were stained for IL-8 and DNA with an IL-8 antibody and Hoechst, respectively (IL-8 in red, *S. flexneri* in green, Hoechst in blue). Inserts show the infection foci (magnification: ×2.5). Scale bars represent 40 μm.

(E) Spatial propagation of IL-8 expression in consecutive A431 or A431-Cx43 bystander cells during *S. flexneri* infection (means ± SD of six wells; graph representative of two independent experiments). Quantification was performed as described in [Supplemental Experimental Procedures](#).

(F) Quantification of bystander IL-8 expression after siRNA-mediated Cx43 depletion. IL-8 was analyzed by immunofluorescence in A431 and A431-Cx43 cells transfected with control or Cx43 siRNAs and infected with *S. flexneri* and quantified by automated image analysis (means ± SD of triplicate wells, graph representative of two independent experiments, $p = 3.9 \times 10^{-6}$).

(G) Quantification of bystander IL-8 expression in A431-Cx43 cells pretreated with 18β-GA and infected with *S. flexneri* (means ± SD of triplicate wells, graph representative of three independent experiments).

(H) Spatial propagation of IL-8 expression from *S. flexneri*-infected A431-Cx43 cells into either A431-Cx43 or A431 adjacent cells. Each number corresponds to the fraction of IL-8-producing cells for a given bystander cell position. Quantification was performed by automated image processing and is described in [Supplemental Experimental Procedures](#) (means ± SD of triplicate wells, graph representative of two independent experiments, $p < 7.5 \times 10^{-4}$ at any given position).

DISCUSSION

In the present study, we provide evidence that during *S. flexneri* infection, the activation of the proinflammatory pathways NF- κ B, JNK, ERK, and p38 propagates from infected to uninfected adjacent cells leading to IL-8 expression in bystander cells of infection. This mechanism, mediated by gap junction communication between infected and uninfected cells, circumvents the immunosuppressive activity of bacterial effectors and massively amplifies inflammation during bacterial infection.

Nod1 contributes to the detection of intracellular *S. flexneri* via the recognition of peptidoglycan-derived peptides (Girardin et al., 2003). In infected IECs, Nod1 ligation leads to NF- κ B activation and upregulation of proinflammatory genes. By using an in vitro single-cell assay of *S. flexneri* infection, we found that NF- κ B activation was not restricted to infected cells. Indeed, by performing infections at low MOIs, we observed within minutes of infection the propagation of NF- κ B activation from infected to uninfected bystander cells. This cell-cell communication mechanism resulted in massive amplification of the total NF- κ B response to *S. flexneri* infection. Because NF- κ B controls the expression of proinflammatory genes, this result suggested that the mechanism of bystander activation may amplify the inflammatory response of an infected epithelial cell layer. This hypothesis was supported by the observation that JNK, ERK, and p38, three kinases involved in the control of inflammation, were also activated in bystander cells of *S. flexneri* infection. Noticeably, p38 activation was markedly higher in bystander than infected cells, suggesting that the effector OspF, which dephosphorylates p38 in the nucleus of infected cells via its phosphothreonine-lyase activity, did not affect p38 bystander activation. This hypothesis was validated by the observation that the deletion of *ospF* enhanced p38 activation in infected but not in bystander cells. Infection at low MOI reflects the early phase of Shigellosis, when the number of bacteria that have reached the basolateral surface of the IECs is limited. By rapidly propagating NF- κ B and MAP kinase activation to uninfected cells, the mechanism of bystander activation may enable the host to fully activate these signaling pathways before their manipulation by future internalized bacteria, for instance via the effectors OspG and OspF (Arbibe et al., 2007; Kim et al., 2005).

The concept of bystander responses has been previously described in the context of ionizing radiation where nonirradiated cells receive signals from neighboring or distant irradiated ones (Hamada et al., 2007), in wound healing (Yang et al., 2004), or more recently after double-stranded DNA recognition (Patel et al., 2009). In all cases, the activation of signaling pathways emanates from cells exposed to local stress and propagates into the adjacent healthy tissue to amplify and orchestrate a multicellular response to an aggression. Here, we provide evidence for a similar mechanism in innate immunity against pathogenic bacteria.

By attracting neutrophils to the infected area, IL-8 has a central function in innate immunity against pathogens and in Shigellosis, in particular. It has been proposed that IL-8 is secreted by *S. flexneri*-infected cells after recognition of intracellular peptidoglycan-derived peptides via Nod1 (Girardin et al., 2003). Yet, the immunosuppressive activity of several effectors that alters

signaling in infected cells challenges the ability of these cells to secrete large amounts of IL-8 as observed in Shigellosis. To directly address this question, we analyzed IL-8 expression during *S. flexneri* infection at the mRNA and protein level by in situ hybridization and intracellular immunofluorescence microscopy, respectively, two methods that combine single-cell resolution and spatial information at the site of infection. Here, we showed that the propagation of NF- κ B, JNK, ERK, and p38 activation leads to IL-8 expression in bystander cells of infection. This mechanism efficiently amplifies the total IL-8 response of the infected cell monolayer by increasing the number of IL-8-producing cells per site of infection. Furthermore, our results clearly demonstrated that infected cells were inefficient at producing IL-8, confirming the immunosuppressive activities of secreted effectors on IL-8 expression. In line with the effect of OspF on p38 and histone H3 phosphorylation, IL-8 production was increased in cells infected with a Δ *ospF* mutant. However, the deletion of *ospF* had only a limited effect on IL-8 expression, suggesting that the complete block observed in infected cells is mediated by multiple effectors. Additional effectors such as OspG, OspB, and IpaH_{9.8} may also contribute to block IL-8 expression in infected cells (Kim et al., 2005; Okuda et al., 2005; Zurawski et al., 2009). Interestingly, we showed that OspF did not affect IL-8 expression in bystander cells, indicating that through cell-cell communication, the host appears to circumvent the activity of OspF in infected cells and amplifies the global IL-8 response to *S. flexneri* infection. In the rabbit intestinal loop model of Shigellosis, IL-8 expression was found in epithelial cells located beyond the zones of bacterial invasion, providing evidence for the physiological relevance of bystander IL-8 expression in vivo (Sansone et al., 1999).

IL-8 expression was also found in bystander cells of *S. typhimurium* and *L. monocytogenes* infection, showing that the potentiation of innate immunity by cell-cell communication corresponded to a broad host response to bacterial infection. However, the contribution of this mechanism to inflammation can vary for different pathogens. It depends on its frequency of occurrence but also on the ability of bacteria to alter signaling in infected cells. For *S. flexneri* that very efficiently blocks signaling in infected cells, bystander activation clearly constitutes the key pathway of IL-8 expression.

We addressed the role of peptidoglycan recognition in the mechanism of cell-cell communication leading to IL-8 expression in bystander cells and its underlying molecular basis. Interestingly, we found that the microinjection of the Nod1 ligand TriDAP was sufficient to induce IL-8 expression in bystander cells of microinjection, suggesting that the recognition of Nod1 ligands in infected cells may be sufficient to generate the underlying signals that mediate IL-8 expression in bystander cells of *S. flexneri* infection. Cell-cell communication can be mediated by different mechanisms: paracrine signaling, direct diffusion of small molecules through gap junctions, or membrane protein interactions. In Caco-2 cells, bystander IL-8 expression was not inhibited by BFA treatment or by perfusion, indicating that this process was most probably not mediated by paracrine signaling. Furthermore, it was cell-cell contact dependent, and therefore compatible with gap junction-mediated communication that enables direct diffusion of small molecules between adjacent

cells. This hypothesis was confirmed by showing that the mechanism of bystander IL-8 expression was blocked by gap junction inhibitors, limited in cells that are poorly coupled via gap junctions, and massively amplified by the overexpression of the gap junction protein Cx43. Finally, as required for the formation of connexins gap junction channels between adjacent cells, we showed that the presence of connexin proteins was necessary in both infected and bystander cells to efficiently propagate inflammation during bacterial infection.

Further studies are required to identify the small molecules (i.e., <2 kDa) diffusing from infected to bystander cells that control NF- κ B, JNK, ERK, and p38 activation and lead to IL-8 expression during *S. flexneri* infection. Among potential candidates, the roles of known second messengers, which are involved in proinflammatory gene expression, including calcium, IP3, and cAMP, should be examined. An alternative hypothesis is the direct diffusion of small peptidoglycan-derived peptides through gap junctions.

In summary, we show that during *S. flexneri* infection, the activation of the proinflammatory pathways NF- κ B, JNK, ERK, and p38 propagates from infected to uninfected adjacent cells leading to IL-8 expression in bystander cells of infection. This mechanism enables the host to circumvent the immunosuppressive activity of bacterial effectors and to massively amplify inflammation during bacterial infection. Moreover, by showing that this process is gap junction mediated, we provide evidence for a direct connection between gap junction communication and amplification of innate immunity during bacterial infection.

EXPERIMENTAL PROCEDURES

Cell Culture

HeLa, A549, Caco-2, A431, and MEF cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and 2 mM L-Glutamine. HUVECs were generously provided by C. Dehio (Biozentrum, University of Basel, Switzerland) and cultivated as previously described (Dehio et al., 1997).

Bacterial Strains

The *S. flexneri* strains M90T wild-type, its noninvasive derivative BS176, and the *icsA* (*virG*) deletion mutant were generously provided by P. Sansonetti (Bernardini et al., 1989). The Δ *virG* Δ *ospF* deletion mutant was generated as described in Supplemental Experimental Procedures (Table S1). The *Salmonella typhimurium* LT2 strain was provided by U. Jenal (Biozentrum, University of Basel, Switzerland) and the stably expressing GFP *Listeria monocytogenes* A21/B5 strain by M. Loessner (ETH Zurich, Switzerland).

Infection Assays

S. flexneri, *S. typhimurium*, and *L. monocytogenes* were used in exponential growth phase. *S. flexneri* and *S. typhimurium* were coated with poly-L-lysine prior to infection. Cells seeded in 96-well plates were infected at indicated MOIs in DMEM supplemented with 10 mM HEPES and 2 mM L-glutamine. After adding bacteria, plates were centrifuged for 5 min and placed at 37°C for indicated time periods. Extracellular bacteria were killed by gentamicin (100 μ g ml⁻¹). Infection assays were stopped by 4% PFA fixation.

Immunofluorescence and IL-8 Measurements

Immunofluorescence and IL-8 measurements were performed as described in Supplemental Experimental Procedures.

Automated Microscopy and Image Analysis

Images were automatically acquired with an ImageXpress Micro (Molecular devices, Sunnyvale, USA). Image analysis was performed via CellProfiler

(Carpenter et al., 2006) and MATLAB (The MathWorks, Inc, Natick, USA) as described in Supplemental Experimental Procedures.

Microinjection and Flow Chamber Experiments

Microinjection and flow chamber experiments are described in Supplemental Experimental Procedures.

Analysis of Cell-Cell Contact

Infection was performed at subconfluent cell density. Cellular contacts and IL-8 were visualized by phalloidin and IL-8 staining, respectively. For each site of infection, the distance between the infected cell and the most distant bystander cell producing IL-8 was used as the radius of the "circle of bystander activation" centered on the infected cell. Within this circle, cells contacting directly the infected cell or indirectly by interacting with other bystander cells were classified as class 1. Cells making no direct or indirect contact with infected cells were defined as class 2. Cells located outside the circle and distant from any sites of infection were defined as class 3.

Statistical Analysis

Data are expressed as mean \pm standard deviation of triplicate samples. p values were calculated with a two-tailed two-sample equal variance t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at doi:10.1016/j.immuni.2010.10.015.

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REFERENCES

- Arbibe, L., Kim, D.W., Batsche, E., Pedron, T., Mateescu, B., Muchardt, C., Parsot, C., and Sansonetti, P.J. (2007). An injected bacterial effector targets chromatin access for transcription factor NF- κ B to alter transcription of host genes involved in immune responses. *Nat. Immunol.* 8, 47–56.
- Bernardini, M.L., Mounier, J., d'Hauteville, H., Coquis-Rondon, M., and Sansonetti, P.J. (1989). Identification of *icsA*, a plasmid locus of *Shigella flexneri* that governs bacterial intra- and intercellular spread through interaction with F-actin. *Proc. Natl. Acad. Sci. USA* 86, 3867–3871.
- Carpenter, A.E., Jones, T.R., Lamprecht, M.R., Clarke, C., Kang, I.H., Friman, O., Guertin, D.A., Chang, J.H., Lindquist, R.A., Moffat, J., et al. (2006). CellProfiler: Image analysis software for identifying and quantifying cell phenotypes. *Genome Biol.* 7, R100.
- Cornelis, G.R. (2006). The type III secretion injectisome. *Nat. Rev. Microbiol.* 4, 811–825.
- Davidson, J.S., Baumgarten, I.M., and Harley, E.H. (1986). Reversible inhibition of intercellular junctional communication by glycyrrhetic acid. *Biochem. Biophys. Res. Commun.* 134, 29–36.
- Dehio, C., Meyer, M., Berger, J., Schwarz, H., and Lanz, C. (1997). Interaction of *Bartonella henselae* with endothelial cells results in bacterial aggregation on the cell surface and the subsequent engulfment and internalisation of the bacterial aggregate by a unique structure, the invasome. *J. Cell Sci.* 110, 2141–2154.

- Dong, C., Davis, R.J., and Flavell, R.A. (2002). MAP kinases in the immune response. *Annu. Rev. Immunol.* 20, 55–72.
- Eckmann, L., Kagnoff, M.F., and Fierer, J. (1993). Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. *Infect. Immun.* 61, 4569–4574.
- Girardin, S.E., Boneca, I.G., Carneiro, L.A., Antignac, A., Jéhanho, M., Viala, J., Tedin, K., Taha, M.K., Labigne, A., Zähringer, U., et al. (2003). Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. *Science* 300, 1584–1587.
- Hamada, N., Matsumoto, H., Hara, T., and Kobayashi, Y. (2007). Intercellular and intracellular signaling pathways mediating ionizing radiation-induced bystander effects. *J. Radiat. Res. (Tokyo)* 48, 87–95.
- Holtmann, H., Winzen, R., Holland, P., Eickemeier, S., Hoffmann, E., Wallach, D., Malinin, N.L., Cooper, J.A., Resch, K., and Kracht, M. (1999). Induction of interleukin-8 synthesis integrates effects on transcription and mRNA degradation from at least three different cytokine- or stress-activated signal transduction pathways. *Mol. Cell. Biol.* 19, 6742–6753.
- Islam, D., Veress, B., Bardhan, P.K., Lindberg, A.A., and Christensson, B. (1997). In situ characterization of inflammatory responses in the rectal mucosae of patients with shigellosis. *Infect. Immun.* 65, 739–749.
- Iwai, H., Kim, M., Yoshikawa, Y., Ashida, H., Ogawa, M., Fujita, Y., Muller, D., Kirikae, T., Jackson, P.K., Kotani, S., and Sasakawa, C. (2007). A bacterial effector targets Mad2L2, an APC inhibitor, to modulate host cell cycling. *Cell* 130, 611–623.
- Kim, D.W., Lenzen, G., Page, A.L., Legrain, P., Sansonetti, P.J., and Parsot, C. (2005). The *Shigella flexneri* effector OspG interferes with innate immune responses by targeting ubiquitin-conjugating enzymes. *Proc. Natl. Acad. Sci. USA* 102, 14046–14051.
- Köhler, H., Rodrigues, S.P., and McCormick, B.A. (2002). *Shigella flexneri* interactions with the basolateral membrane domain of polarized model intestinal epithelium: Role of lipopolysaccharide in cell invasion and in activation of the mitogen-activated protein kinase ERK. *Infect. Immun.* 70, 1150–1158.
- Kufer, T.A., Banks, D.J., and Philpott, D.J. (2006). Innate immune sensing of microbes by Nod proteins. *Ann. N Y Acad. Sci.* 1072, 19–27.
- Lee, J., Mira-Arbibe, L., and Ulevitch, R.J. (2000). TAK1 regulates multiple protein kinase cascades activated by bacterial lipopolysaccharide. *J. Leukoc. Biol.* 68, 909–915.
- Li, H., Xu, H., Zhou, Y., Zhang, J., Long, C., Li, S., Chen, S., Zhou, J.M., and Shao, F. (2007). The phosphothreonine lyase activity of a bacterial type III effector family. *Science* 315, 1000–1003.
- Makino, S., Sasakawa, C., Kamata, K., Kurata, T., and Yoshikawa, M. (1986). A genetic determinant required for continuous reinfection of adjacent cells on large plasmid in *S. flexneri* 2a. *Cell* 46, 551–555.
- Mollenhauer, H.H., Morré, D.J., and Rowe, L.D. (1990). Alteration of intracellular traffic by monensin; Mechanism, specificity and relationship to toxicity. *Biochim. Biophys. Acta* 1031, 225–246.
- Neijssen, J., Herberts, C., Drijfhout, J.W., Reits, E., Janssen, L., and Neefjes, J. (2005). Cross-presentation by intercellular peptide transfer through gap junctions. *Nature* 434, 83–88.
- Ninomiya-Tsuji, J., Kishimoto, K., Hiyama, A., Inoue, J., Cao, Z., and Matsumoto, K. (1999). The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway. *Nature* 398, 252–256.
- Okuda, J., Toyotome, T., Kataoka, N., Ohno, M., Abe, H., Shimura, Y., Seyedarabi, A., Pickersgill, R., and Sasakawa, C. (2005). *Shigella* effector IpaH9.8 binds to a splicing factor U2AF(35) to modulate host immune responses. *Biochem. Biophys. Res. Commun.* 333, 531–539.
- Parsot, C. (2009). *Shigella* type III secretion effectors: How, where, when, for what purposes? *Curr. Opin. Microbiol.* 12, 110–116.
- Patel, S.J., King, K.R., Casali, M., and Yarmush, M.L. (2009). DNA-triggered innate immune responses are propagated by gap junction communication. *Proc. Natl. Acad. Sci. USA* 106, 12867–12872.
- Pédron, T., Thibault, C., and Sansonetti, P.J. (2003). The invasive phenotype of *Shigella flexneri* directs a distinct gene expression pattern in the human intestinal epithelial cell line Caco-2. *J. Biol. Chem.* 278, 33878–33886.
- Phalipon, A., and Sansonetti, P.J. (2007). *Shigella*'s ways of manipulating the host intestinal innate and adaptive immune system: A tool box for survival? *Immunol. Cell Biol.* 85, 119–129.
- Saccani, S., Pantano, S., and Natoli, G. (2002). p38-dependent marking of inflammatory genes for increased NF-kappa B recruitment. *Nat. Immunol.* 3, 69–75.
- Sansonetti, P.J., Arondel, J., Huerre, M., Harada, A., and Matsushima, K. (1999). Interleukin-8 controls bacterial transepithelial translocation at the cost of epithelial destruction in experimental shigellosis. *Infect. Immun.* 67, 1471–1480.
- Schroeder, G.N., and Hilbi, H. (2008). Molecular pathogenesis of *Shigella* spp.: Controlling host cell signaling, invasion, and death by type III secretion. *Clin. Microbiol. Rev.* 21, 134–156.
- Strober, W., Murray, P.J., Kitani, A., and Watanabe, T. (2006). Signalling pathways and molecular interactions of NOD1 and NOD2. *Nat. Rev. Immunol.* 6, 9–20.
- Troyanovsky, S.M., Troyanovsky, R.B., Eshkind, L.G., Krutovskikh, V.A., Leube, R.E., and Franke, W.W. (1994). Identification of the plakoglobin-binding domain in desmoglein and its role in plaque assembly and intermediate filament anchorage. *J. Cell Biol.* 127, 151–160.
- Wang, C., Deng, L., Hong, M., Akkaraju, G.R., Inoue, J., and Chen, Z.J. (2001). TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature* 412, 346–351.
- Yang, L., Cranson, D., and Trinkaus-Randall, V. (2004). Cellular injury induces activation of MAPK via P2Y receptors. *J. Cell. Biochem.* 91, 938–950.
- Zurawski, D.V., Mummy, K.L., Faherty, C.S., McCormick, B.A., and Maurelli, A.T. (2009). *Shigella flexneri* type III secretion system effectors OspB and OspF target the nucleus to downregulate the host inflammatory response via interactions with retinoblastoma protein. *Mol. Microbiol.* 71, 350–368.